# Construction and Screening of a cDNA Library to Isolate Chicken Pituitary Hormone Genes[1]

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ABSTRACT. A chicken pituitary cDNA expression library has been constructed in the bacteriophage vector λgt 11 (methods for the bacteriophage DNA propagation, modification, ligation of the double-stranded cDNA insert and *in vitro* packaging are included.) The chicken pituitary cDNA library was screened with heterologous pituitary cDNA clones, and several putative chicken specific pituitary cDNA clones were isolated. These putative chicken specific cDNAs should prove most useful in determining the structural organization of the various pituitary hormone genes and help to better understand the molecular mechanisms of how these sequences affect growth, reproduction, and metabolism in the chicken. For educational purposes, the techniques of cDNA library construction and screening should allow advanced undergraduate students the opportunity to isolate specific cDNA clones of interest. After learning such methodologies, students should be in an advantageous position in the promising job market for recombinant DNA technology and for admission to the best graduate schools in the areas of cellular and molecular biology.

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## INTRODUCTION

Complementary DNA (cDNA) synthesized from poly $A^+$-containing, messenger RNA (mRNA) transcripts can be used to construct cDNA libraries in plasmid or bacteriophage vectors. Such cDNA libraries are useful for isolating and cloning specific cDNA sequences using homologous as well as heterologous probes. Since cDNA libraries contain only sequences that are actively transcribed (as opposed to genomic libraries that theoretically contain all the sequences of a particular genome), the relative abundance levels of the various mRNAs will be reflected in the representative number of recombinant cDNA molecules present in the library. Less than half the mass of the mRNA in a cell consists of a large number of mRNA sequences (10,000 or more), each of which is represented by a small number of mRNA copies per cell (Lewin 1980). These low abundance mRNA molecules may only be present in 10 copies per cell and constitute about 0.005% or less of the mass of total cellular mRNA. In order to isolate a desired low abundance cDNA clone, it would be necessary to screen $10^6$-$10^7$ members (i.e., bacteriophage plaques containing specific cDNA inserts) of a recombinant cDNA library. For isolating middle abundant mRNA molecules (approximately $10^2$-$10^4$ copies per cell or 0.05% to 5% of the mass of total mRNA), $10^4$-$10^6$ recombinant members would have to be screened. On the other hand, high abundance mRNA molecules (i.e., $10^4$-$10^5$ copies per cell or 5-50% of the total mRNA mass) would only require screening $10^3$-$10^4$ recombinant members. Due to their increased representation in a cDNA library, a number of high abundance class mRNA molecules have been isolated and cloned. There is no correlation between the abundance level of a particular mRNA species in a cell and the copy number of that corresponding gene. Examples of single copy genes that are highly transcribed include the ovalbumin gene (Harris et al. 1973), human γ globin genes (Old et al. 1976), silk fibroin (Suzuki et al. 1972), and δ crystallin (Zelenka and Piatgorsky 1976).

Complementary DNA libraries can be generated in expression or non-expression type vectors depending on their anticipated use. Procedures for constructing cDNA libraries that use non-expression vectors include, for example, cDNA "tailing" (Villa-Komaroff et al. 1978) and direct cloning of cDNA (Okayama and Berg 1982) into plasmid vectors, as well as cloning cDNA inserts into bacteriophage such as λgt 10 (Huynh et al. 1985, Huynh and Davis unpublished data). Recombinant, non-expression type vectors can be probed with homologous and heterologous cDNA or genomic nucleic acid probes.

Expression type vectors use a transcriptional promoter located upstream from the cDNA insert. Often these promoters can be induced by various mitogens, chemical reagents, or physical means to greatly increase the copy number of the transcripts. Recombinant, expression type vectors can be probed with homologous and heterologous cDNA or genomic nucleic acid probes. In addition, some expression vectors such as the bacteriophage λgt 11 (Young and Davis 1983), by virtue of their ability to synthesize a gene product in the form of a foreign antigen, have the major advantage that they can be screened with monoclonal or polyclonal antibodies in order to isolate and clone genes of interest.

A recombinant cDNA expression library or gene bank has been prepared in the phage vector λgt 11 that contains cDNA inserts synthesized from chicken pituitary poly $A^+$ mRNA. This library was constructed for the purpose of isolating cDNA clones for a number of the chicken pituitary hormone genes (i.e., growth hormone and prolactin, as well as thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicle stimulating hormone (FSH)). The latter three hormones have a common α and distinct β subunits and are post-translationally modified (i.e., by glycosylation and the addition of sialic acid residues). The study of these chicken pituitary hormone genes will lead to analysis of their structural organization, their regulation of expression,

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and their role in growth, reproduction, and metabolism *in vivo*.

The approaches outlined can be used as workable methodologies for educating undergraduates in the cellular molecular biology area. The technologies are not merely limited to the larger research laboratories since many of the procedures actually have been simplified and optimized as molecular biology reaction kits for the various parts of the cDNA library construction. By introducing the techniques and terminology of recombinant DNA molecular biology, undergraduates will be better prepared for the expanding job market in biotechnology or acceptance into the better cellular and molecular biology graduate school programs.

## MATERIALS AND METHODS

VECTOR PREPARATION. The bacterophage vector λgt 11 was grown in *Escherichia coli* BNN97 in T broth (10 g tryptone, 5 g NaCl, 2 g maltose, 10mM $MgSO_4$ per L) at 32°C until reaching 0.4 OD units ($A_{590}$). The phage preparation was heat-shocked for 15 min at 42°C to induce lysis, and was then grown overnight at 37°C shaking. The phage were purified essentially as described in Maniatis et al. (1982). The purified λgt 11 DNA was cut with restriction endonuclease *Eco* R1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) followed by phosphatase treatment. This latter step prevents self-religation of the vector and greatly increases the number of recombinant molecules. (For best results we use 2-3 units of Boehringer Mannheim, molecular biology-grade, calf intestine, alkaline phosphatase per 100 μg DNA per 30-min reaction at 37°C, followed by heat inactivation at 68°C for 15 min, phenol extraction twice, and a final chloroform extraction before ethanol precipitation of the λgt 11 DNA.)

cDNA INSERT SYNTHESIS AND MODIFICATION. Approximately 300 pituitaries from adult layer hens were isolated and immediately frozen on dry ice. Tissues (approximately 2.1 g) were dispersed by pulverizing in the presence of liquid nitrogen followed by homogenization using essentially the guaninidium isothiocyanate methods of Chirgwin et al. (1979). The pellet of total cellular RNA was resuspended and passed over oligo dT cellulose (Aviv and Leder 1972) to obtain total cellular poly $A^+$ mRNA (yielding 11.5 μg poly $A^+$ mRNA). The tails of the isolated poly $A^+$ mRNA were primed with 1.0 μg oligo dT followed by single strand cDNA synthesis with 2-3 units avian myeloblastosis virus reverse transcriptase per μg poly $A^+$ mRNA (J. Beard, Life Sciences, Inc., Sarasota, FL). Synthesis was in the presence of $^{32}P$ α dCTP in a 500:1 ratio of unlabeled to radiolabeled nucleotide using essentially the procedure of Buell et al. (1978). The Klenow fragment of DNA polymerase I (Boehringer Mannheim Biochemicals) was used to synthesize the second strand of cDNA; the hairpin loop was removed by S1 nuclease treatment. The 11.5-μg, poly $A^+$ mRNA template yielded 14 μg of double-stranded cDNA (before S1 treatment) calculated by incorporated $^{32}P$ α dCTP.

The size of the double-stranded cDNA product is shown in Figure 1. This figure shows an autoradiogram of chicken oviduct (prepared for another library to isolate the chicken avidin gene) and pituitary double-stranded cDNA (pre- and post-S1 nuclease treated), resolved by agarose gel electrophoresis, dried, and exposed to Kodak XAR-5 film. An approximate band of 1900 base pairs (bp) that represents full length ovalbumin cDNA can be seen in the second lane (S1-treated oviduct cDNA). This indicates that full length, cDNA synthesis was achieved for a large number of cDNA molecules. As shown, the average size of the S1 treated pituitary cDNA is approximately 650 bp.

To ensure blunt ends, the double-stranded cDNA was treated with the Klenow fragment of *E. coli* polymerase 1, then ligated to *Eco* R1 linkers (Boehringer Mannheim Biochemicals) according to the protocols of Maniatis et al. (1982). After digestion with *Eco* R1, the linkered cDNA was purified by Sepharose 4B gel filtration. At this point the cDNA was size-fractionated to ensure that only the longest molecules were cloned. The *Eco* R1 linkered, double-stranded cDNA was ligated to purified λgt 11 vector that had been cleaved with *Eco* R1 and phosphatased. The recombinant molecules were packaged with *in vitro* packaging kits (Amersham Corp., Arlington Heights, IL) and grown in *E. coli* Y1088 to generate a cDNA library. The $3 \times 10^{10}$ plaque-forming units in the library contained $7 \times 10^6$ individual members of which 76% were recombinant (as scored by white or blue plaques with the chromagenic indicator X gal and isopropyl β D-thiogalactopyranoside).

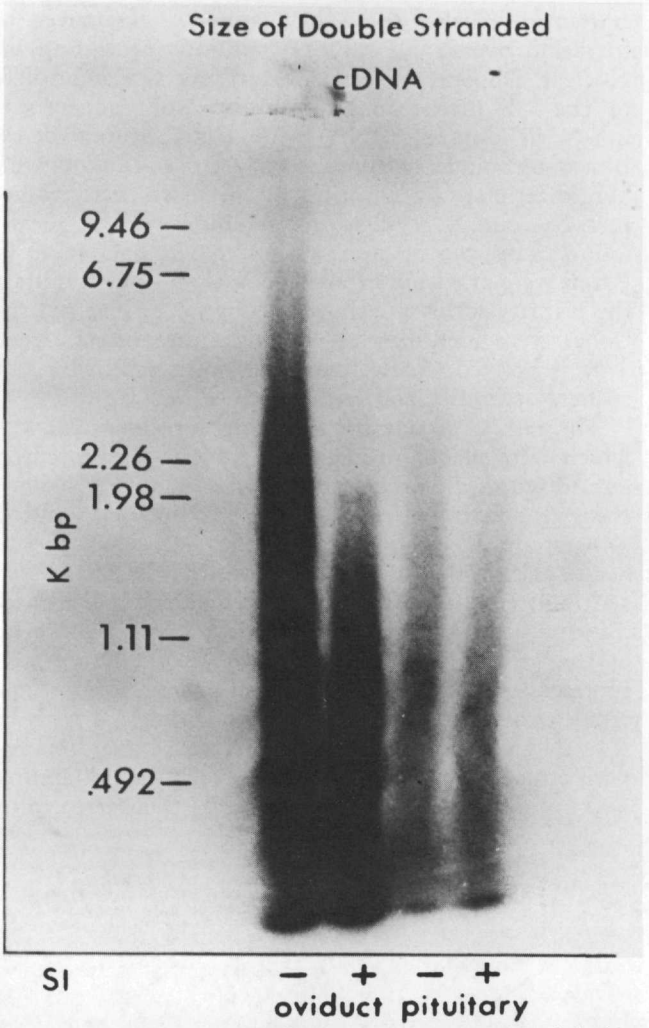


FIGURE 1. Autoradiogram of agarose gel showing size of double-stranded cDNA from oviduct and pituitary poly $A^+$ mRNA pre- and post-S1 nuclease treatment.

## RESULTS

To screen the library, approximately $10^6$ members of the cDNA library (enough members to detect a RNA abundance level of 50-100 transcripts) were adsorbed to *E. coli* Y1090. After a 15-min incubation, the mixture was added to 7 ml of melted, 0.7%, low-gelling, temperature agarose in T broth and plated onto 20 T broth plates and incubated overnight at 37°C. After allowing the top agarose to harden for 1 h at 4°C, two replica nitrocellulose filters were made per plate with modifications of the Benton and Davis (1977) technique. Filters were denatured in NaOH, neutralized, then baked and prepared for hybridization with essentially the procedures of Jeffries and Flavell (1977).

The filters were probed with a cloned, heterologous, bovine TSHβ cDNA (clone 24-7). The bTSHβ cloned DNA was first transfected into $recA^-$ HB 101 cells under antibiotic selection and then grown in mass. The cloned plasmid DNA was digested with *Pst* I and electrophoresed on a 1.0% agarose gel. The 531-bp bTSH insert was cut out of the gel. The DNA was removed from agarose by electroelution and recovered after ethanol pre-

cipitation. Further traces of agarose were removed by filtration over a NENSORB column (New England Nuclear, Dupont, Inc.). Replica filters were hybridized to the $^{32}P$ nick-translated probe (specific activity 2-5 $\times$ $10^8$ dpm/ug DNA) with a modification of the Blotto technique of Johnson et al. (1984). Following hybridization at 42°C for 24 h, the filters were washed in 2x SSC and 0.1% SDS at room temperature for 30 min followed by two 30-min washes in .2x SSC at 55°C. Filters were dried and exposed to XAR-5 film. Results of the primary screen are shown in Figure 2. Five positive clones were identified on the duplicate filters out of 1 $\times$ $10^6$ plated. These five clones were picked for subsequent secondary and tertiary screening.

The results of tertiary screening for clones 2 and 3 which were plaque-purified (i.e., every plaque present hybridized to the probe) are shown in Figure 3. Four of the five positive primary clones were plaque-purified.

A single plaque from one of the four clones was selected and grown in mass in T broth. The phage were sedimented after DNase 1, RNase A, and RNase T1 digestion and then lysed after heat inactivation of the DNase. The cloned DNA was digested with proteinase K, extracted twice with phenol, and precipitated with ethanol. Cloned DNA was digested with *Eco* R1, electrophoresed on a 1% agarose gel, and stained with ethidium bromide. A single *Eco* R1 band (approx. 380 bp) was observed. The DNA was transferred to nitrocellulose paper according to Jeffries and Flavell (1977), baked and hybridized for 24 h to the nick-translated bTSH$\beta$ probe according to Johnson et al. (1984). Following washing at 55°C in .2x SSC, the filter was dried and exposed to X-ray film. Audioradiography (data not shown) revealed that the digested, putative chicken TSH$\beta$ cDNA-cloned insert hybridized to the heterologous, bTSH$\beta$ probe.

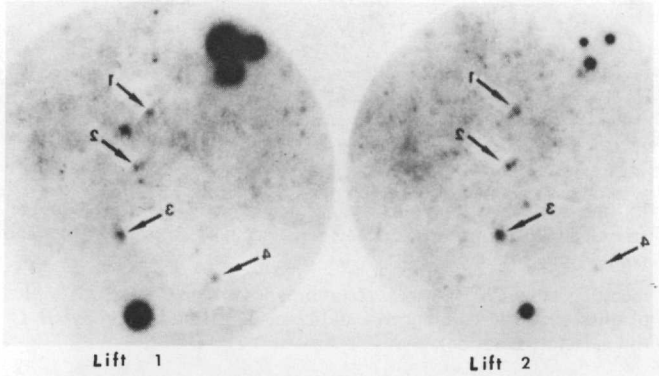


FIGURE 2. Autoradiogram of primary screening of chicken pituitary cDNA expression library. Duplicate filters were adsorbed to approximately 5 $\times$ $10^4$ plaques and processed for hybridization as described in the text. Filters were probed with $^{32}P$-labeled bTSH$\beta$ cDNA insert using the "Blotto" technique followed by washing at high stringency (55°C, 2 $\times$ SCC), drying, and exposure to X-ray film.

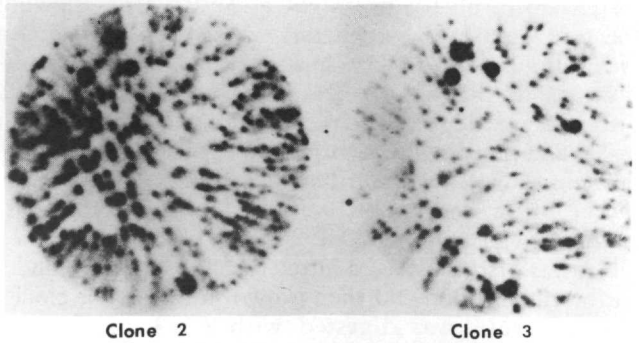


FIGURE 3. Autoradiogram of tertiary screening of two different clones from the chicken pituitary cDNA expression library. All plaques hybridize, suggesting sequences have been plaque-purified. Hybridization conditions as in Fig. 2.

## DISCUSSION

One of the goals of this laboratory is to better understand what regulates the growth, reproduction, and metabolism of the chicken. Toward this goal we have begun isolating a number of the chicken pituitary hormone genes in order to define their organization and to determine by what mechanisms the gene products of these sequences regulate a number of physiological processes in the bird. Also, we are interested in learning more about the regulation of pituitary hormone gene expression *in vitro* and in the animal. The construction of a cDNA library from chicken pituitary poly $A^+$ mRNA has served as a first step for isolating and cloning a number of the pituitary hormone genes. We have isolated putative chicken pituitary hormone cDNA clones from the recently constructed library and will be proving their identity by sequence analysis. Once the various chicken pituitary hormone genes and their cDNAs have been isolated and characterized, we hope to produce these hormones in large amounts by recombinant DNA methodologies. For the first time we should have enough purified, biologically active material to assess the effects of these molecules in the chicken. It is hoped that through such techniques as site-directed mutagenesis, more potent pituitary hormones may be produced, and that a more efficient animal in terms of growth and metabolism might result.

## RELEVANCE OF TECHNIQUES TO UNDERGRADUATE EDUCATION

The recombinant DNA methodologies that have been outlined are also well suited for use in a number of advanced undergraduate courses and senior honors projects. These techniques at one time were appropriate only for graduate and post-doctoral investigators, but now in many instances undergraduates are expected to have a working knowledge of such procedures before entering graduate school or the technical job market.

The principles of cDNA library construction and the isolation of specific cDNA sequences using antibody or nucleic acid probes can be taught in undergraduate courses in genetics and molecular biology, or in a seminar on laboratory techniques. In addition, the "hands-on" laboratory techniques outlined can be most beneficial for students that are pursuing a senior honors project involving laboratory work. The techniques lend themselves well to usage by the individual student or groups of students. There are also many "stopping points" along the way, so that if one group of undergraduates can complete only a certain portion of the library construction or screening, another group can continue on toward the completion of the project at a later time.

During the course of working with these recombinant DNA methodologies, students are exposed to a variety of basic molecular techniques. When a solid undergraduate

biology and chemistry background is combined with a knowledge of some or all of the techniques involved with cDNA library construction and gene cloning, students will also have a distinct advantage over those devoid of such training for the very competitive positions in molecular genetics and biochemistry departments in our best graduate schools. If these students do not wish to pursue post-graduate education, their training will allow them to compete effectively in the technical job market. It has been estimated that by the year 1990 some 37,000 jobs involving some aspect of recombinant DNA technology will be available. This number will undoubtedly increase greatly by the year 2000 (U.S. Dept. of Health and Human Services 1985), both in industrial and academic settings. There is already a demand for well trained technicians in industry and academic institutions. The employment future for such individuals is indeed bright.

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